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Implication of Protein Kinase B/Akt and Bcl-2/Bcl-XL Suppression by the Farnesyl Transferase Inhibitor SCH66336 in Apoptosis Induction in Squamous Carcinoma Cells

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Abstract

The farnesyltransferase inhibitor SCH66336 exhibits antitumor activity in vitro and in vivo; however, its mechanism of action is still unresolved. We found that SCH66336 suppressed growth and induced apoptosis of human head and neck squamous carcinoma cells (HNSCC). SCH66336 suppressed protein kinase B/Akt activity as well as the phosphorylation of the Akt substrates glycogen synthase kinase (GSK)-3β, forkhead transcription factor, and BAD. Infection of SqCC/Y1 cells with an adenovirus that contained a constitutively active form of Akt rescued cells from SCH66336-induced apoptosis. These results suggest that SCH66336 is a potent apoptosis inducer in HNSCC cells and that it may act by suppressing the Akt pathway.

Introduction

Protein prenylation is a posttranslational modification in which either a farnesyl or a geranylgeranyl isoprenoid is linked via a thioether bond to specific cysteine residues of proteins. These proteins belong to a group termed “CaaX proteins,” which is defined by a specific COOH-terminal motif that directs their modification. The CaaX-type prenylated proteins that are found primarily at the cytoplasmic face of cellular membranes include Ras and a multitude of GTP-binding proteins, several protein kinases and phosphatases, proteins CENP-E and CENP-F, and nuclear laminas (1). Protein farnesylation is controlled by the enzyme FTase. The understanding of the farnesylation reaction and of the substrate specificity of FTase has led to the rational design of several different FTIs. FTIs have demonstrated significant antitumor activity against experimental models of human cancer (2–4). SCH66336 {(+)-2-[4-(8-chloro-3,10-dibromo-6,11-dihydro-5H-benzo[(5,6)-cyclohepta[1,2-b]-pyridin-11(6H)]-yl)-1-piperidinyl]-2-oxo-ethyl]-1-piperidinecarboxamide}, a potent nonpeptide tricyclic FTI (5), was one of the first FTIs to be tested in mice. SCH66336 inhibited the anchorage-independent growth of many human tumor lines irrespective of whether they contained a wild-type or an activated ras oncogene (7, 8). In vivo, SCH66336 demonstrated potent oral activity against a wide array of human tumor xenograft models (colon, lung, pancreas, prostate, and urinary bladder) in nude mice. Oral SCH66336 treatment, initiated after mice had developed palpable tumors, caused tumor regression via decreased DNA synthesis and increased apoptosis (7). Most FTIs uncouple Ras activity, but they also inhibit the growth of transformed cells in vitro and exhibit antitumor activity in vivo in the absence of ras mutations. Therefore, farnesylated proteins other than ras may contribute to the action of FTIs. Clearly, additional investigations on the mechanism by which SCH66336 exerts its antitumor activities are warranted.

In the present report, we describe the ability of SCH66336 to decrease the survival and induce apoptosis of head and neck cancer cells by suppressing the activity of PKB, also known as Akt, a phosphoprotein substrate of PI3K, which is involved in the regulation of cell proliferation and survival and which is an excellent target for novel cancer therapies (9).

Materials and Methods

Cells and Culture Conditions

Human HNSCC cell lines UMSCC10B, UMSCC14B, UMSCC17B, UMSCC22B, and UMSCC35, UMSCC38 cell lines were obtained from Dr. T. Carey (University of Michigan, Ann Arbor, MI). HNSCC cell lines 183A and 1483 were provided by Dr. P. G. Sacks (New York University College of Dentistry, New York, NY). SqCC/Y1 cells were provided by Dr. M. Reiss (Yale University, New Haven, CT). TR146 cells were provided by Dr. A. Balm (The Netherlands Cancer Institute, Amsterdam, The Netherlands). These cells were grown in monolayer culture in a 1:1 (v/v) mixture of DMEM and Ham’s F12 medium, supplemented with 5% fetal bovine serum and antibiotics, at 37°C in a humidified atmosphere consisting of 95% air and 5% CO2.

Cell Survival Assays

The cells were seeded in 96-well cell-culture cluster plates at a density that allowed control cultures to grow exponentially for 5 days. After 24 h, the cells were treated with different concentrations of SCH66336 (provided by Schering-Plough Research Institute, Kenilworth, NJ). SCH66336 was dissolved in DMSO. Control cultures received the same amount of DMSO as the treated cultures did. Cell numbers were estimated after 5 days of treatment by SRB assay, as described previously (10). The percentage of growth inhibition was calculated by using the equation: percentage growth inhibition = (1 – At/Ac) X 100, where At and Ac represent the absorbance in treated and control cultures, respectively. The drug concentration causing a 50% cell growth inhibition (IC50) was determined by interpolation from dose-response curves.

Cell Cycle Analysis

Cells treated for 24, 48, or 72 h, with either SCH66336 or DMSO (control), were harvested and fixed in 70% cold ethanol. Cells were stored at 4°C overnight. The cells were then stained with propidium iodide (50 µg/ml) in a
buffer containing 50 μg/ml Rnase and were then stored at 4°C overnight before analysis. DNA content was measured using an EPICS 752 flow cytometer (Coulter Corporation, Hialeah, FL). Data analysis was performed using “Multi” series (Phoenix Flow Systems, San Diego, CA) and Summit (Cytometry) software.

**Anchorage-independent Growth Assay.** HNSCC cells were mixed in low-temperature melting agarose (0.5%) and then placed on top of solidified agarose (1%) in 6-well plates at 2000 cells/well. Both bottom and top agarose layers contained either 0.01% DMSO (as a solvent control) or different SCH66336 concentrations. After the cell-containing top agarose layer was allowed to solidify in a 4°C cold room, the dishes were incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C for 14 days. DMEM plus 5% FBS (with or without SCH66336; 0.5 ml) was added on top of the agarose after 3 days and replaced every 3 days thereafter. At the end of the experiments, the colonies were counted under an inverted microscope at ×40.

**Apoptosis Assays**

**DNA Ladder Formation.** Cells were grown in the absence or presence of SCH66336 (1 μM) for 1–5 days. After 24 h and at 24-h intervals thereafter, floating and attached cells were collected and lysed. Soluble DNA was extracted with phenol-chloroform, precipitated in ethanol, and electrophoresed on a 1.8% agarose gel. The gels were then stained with ethidium bromide and photographed in the dark using UV illumination.

**TUNEL.** Cells were plated on either 24-well plates or 10-cm diameter dishes 1 d before treatment. Apoptosis was evaluated by the TUNEL assay daily after 1–5 days of treatment using the APO-DIRECT kit (Phoenix Flow Systems, Inc., San Diego, CA) following the manufacturer’s protocol. Flow cytometric analysis was conducted using a Coulter EPICS Profile II flow cytometer (Coulter Corp., Miami, FL). Approximately 10,000 events (cells) were evaluated for each sample. Gating of control populations (cells treated with DMSO only) was used as a reference to compare with treatments with SCH66336. The percentage of apoptosis was determined from the proportion of FITC-positive cells within the 10,000 cells analyzed.

**Protein Extraction and Western Blot Analysis**

Cells were washed in PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. After incubation on ice for 15 min and centrifugation at 12,000 rpm for 10 min, the supernatants were collected and protein concentration was determined using a Protein Assay Kit (Bio-Rad, Hercules, CA). Protein (30 μg) was electrophoresed through a 12% polyacrylamide gel and transferred to a nitrocellulose membrane by electroblotting. Membranes were probed with antibodies, and antibody binding was detected using an enhanced chemiluminescence (ECL) kit (Amersham Life Science, Arlington Heights, IL) according to the manufacturer’s directions.

The following antibodies were used for Western blotting. Akt antibody (no. 9272), phospho-Akt (Ser473) antibody (no. 9271), phospho-p44/42 MAP kinase ERK1/2 (Thr202/Tyr204) E10 monoclonal antibody (no. 9106), p44/42 MAP kinase antibody (no. 9102), phospho-GSK-3 (Ser9) antibody (no. 9272), phospho-Akt (Ser473) antibody (no. 9271), phospho-p44/42 MAP kinase antibody (no. 9106), p44/42 MAP kinase antibody (no. 9102), phospho-GSK-3β (Ser9) antibody (no. 9336), phospho-FKHR (Ser256) antibody (no. 9461), phospho-Bad (Ser136) (HD11) were from Santa Cruz Biotechnology. Mouse polyclonal anti-anti-Fas antibody (C-20; SC-715), and mouse monoclonal anti-Bad antibody (C-20) were from Dako Corp. Rabbit polyclonal anti-Bcl-x/L antibody (S-18), rabbit polyclonal anti-Bax antibody, mouse monoclonal anti-Bcl-2 antibody (C-4), and mouse monoclonal anti-Bcl-x/L antibody (C-20) were from Santa Cruz Biotechnology. Mouse polyclonal anti-caspase-3 antibody and mouse monoclonal anti-Bax antibody were obtained from Sigma Chemical Company (St. Louis, MO). Purified mouse anti-human Fas ligand monoclonal antibody (4556387) and purified mouse anti-human TRAIL monoclonal antibody (no. 556468) were obtained from BD Pharmingen (San Diego, CA). Membranes were reprobed with anti-actin antibodies as controls for loading in each lane. The level of proteins relative to actin was calculated by image analysis using the NIH Image program.

**Generation of AdsCMV-HA-Myr-Akt**

An adenoviral vector expressing a full-length human Akt1 with the Src myristylation signal fused in-frame to the c-Akt coding sequence with HA (11) under the control of CMV promoter (AdsCMV-MyrAkt-HA) was constructed using the pAd-shuttle vector system, as described previously (12). The presence of MyrAkt-HA was confirmed by dye-deoxy-DNA sequencing and Western blot analysis on Akt and HA. The activity of AdsCMV-MyrAkt-HA was examined by a Western blot analysis on pGSK-3β (Ser 9). Viral titers were determined by plaque assays and spectrophotometric analysis.

**Results**

**SCH66336 Suppresses Anchorage-dependent and Anchorage-independent Growth of HNSCC Cells.** Treatment of 10 different HNSCC cell lines with SCH66336 at doses ranging from 0.1 to 8 μM resulted in growth suppression that was dose dependent and time dependent. The results obtained with SqCC/Y1 cells are presented as an example in Fig. 1A. The different cell lines exhibited a range of sensitivities to SCH66336 with three cell lines (TR146, SqCC/Y1, and UMSSC35) very sensitive (IC₅₀ values after a 5-day treatment were 0.43, 0.45, and 0.58 μM, respectively) with six cell lines (UMSSC38, 183, UMSCC22B, UMSCC17B, UMSCC14B, and UMSCC10B) partially sensitive (IC₅₀ values were 2.0, 2.6, 2.8, 2.9, 4.4, and 4.4 μM, respectively), and with one cell line (1483) being resistant to 8 μM SCH66336 (data not shown).

Cell cycle analyses performed with the SqCC/Y1 cells revealed that SCH66336 treatment for 2 or more days resulted in a drop in G₁ cell population and an increase in the G₂-M population. After 3 days, there was also an increase in the sub-G₁ population and decreases in the S-phase and G₂-M populations (Fig. 1B).

The ability of the HNSCC cells to form colonies in semisolid agarose (anchorage-independent growth) was compromised by SCH66336 in a dose-dependent fashion with IC₅₀ of ~1.6 μM (Fig. 1C). The colony-forming ability of five other HNSCC cell lines was also inhibited by SCH66336 as follows: UMSCC22B, TR146, and 183A had IC₅₀ values of 1.0, 1.7, and 4 μM, respectively. Colony formation by UMSCC17B and UMSCC38 was inhibited by about 40% at 4 μM SCH66336 (data not shown).

**SCH66336 Induces Apoptosis in SqCC/Y1 Cells.** To determine whether the decrease in cell survival was mediated by induction of apoptosis, we analyzed the effect of 1 μM SCH66336 on DNA fragmentation in six HNSCC cell lines using the TUNEL assays after 1–5 days of treatment. The cells lines exhibited a time-dependent induction of apoptosis albeit with various degrees of sensitivity to SCH66336. After 5 days of treatment, percentages of apoptosis in SqCC/Y1, TR146, UMSCC22B, 183A, UMSSC38, and UMSCC17B were 89, 45, 40, 30, 15, and 13%, respectively.

The proapoptotic effects of SCH66336 were examined further with the SqCC/Y1 cells, using several experimental approaches: the TUNEL assay (Fig. 2A), DNA ladder formation (Fig. 2B), and caspase-3 activation/PARP cleavage (Fig. 2C). All of these methods have shown that apoptosis is induced 2–3 days after exposure to SCH66336. The TUNEL data indicate that apoptosis was induced in ~30% of the cells on day 3 and in nearly 90% by day 5 (Fig. 2A). The level of the procaspase-3 was diminished by day 2, indicating activation of this caspase. The finding that its substrate PARP was cleaved after 3 days of exposing SqCC/Y1 cells to SCH66336 further supports the contention that caspase-3 was activated (Fig. 2C).

**SCH66336 Decreases the Levels of Some Members of the Bcl-2 Family.** To begin to elucidate the components of the pathway involved in apoptosis, we analyzed the levels of several members of the Bcl-2 family of proteins. We found that the levels of the antiapoptotic proteins Bcl-2 and Bcl-XL declined progressively beginning 1–2 days after exposure of the cells to 1 μM SCH66336, whereas the levels of the antiapoptotic protein Bax remained unaltered (Fig. 2D and 2E).

Thus, the ratio of the proapoptotic protein to the antiapoptotic proteins increased substantially (Fig. 2E).
SCH66336 Suppresses the Level of Akt Protein, Its Phosphorylated Form, and Downstream Molecules. Because Akt is a major regulator of cell survival, we examined its level and phosphorylation state in 10 HNSCC cell lines. The levels of Akt detected by Western blotting were high in UMSCC14B, UMSCC35, SqCC/Y1; intermediate in 1483, UMSCC38, and 183A; and low in TR146, UMSCC22B, UMSCC17B, and UMSCC10B. The levels of phosphorylated Akt (pAktS473) were high in UMSCC14B, SqCC/Y1, 1483, TR146, UMSCC17B, UMSCC22B; intermediate in UMSCC35 and UMSCC10B; and low in UMSCC38 and 183A.

We then examined the effects of SCH66336 on Akt status and function in SqCC/Y1 cells, which were both sensitive to SCH66336 and which expressed high levels of Akt and pAkt(S473) as well. The amount of both Akt1 and Akt2 proteins declined in SCH66336-treated cells albeit with different kinetics. Whereas Akt1 decreased by 12 to 18 h after treatment initiation, Akt2 began to diminish after 24 to 36 h. The decrease in Akt1 was more pronounced, with almost complete disappearance of this protein after 24 h of treatment, whereas Akt2 levels were low but detectable after 48 h (Fig. 3A). An analysis of the levels of phosphorylated Akt also showed a decrease in SCH66336-treated cells 18 h after treatment when total Akt protein level was still detectable (Fig. 3B). The levels of phospho-Akt (pAktS473) were high in UMSCC14B, SqCC/Y1, 1483, TR146, UMSCC17B, UMSCC22B; intermediate in UMSCC35 and UMSCC10B; and low in UMSCC38 and 183A.

The expression levels of the proteins GSK-3β, FKHR, and Bad were not altered after SCH66336 treatment for up to 48 h. However, the level of their phosphorylated forms diminished after 3 h (p-GSK-3β Ser9), 12 h (p-FKHR Ser256), and 24 h (p-Bad Ser136), respectively (Fig. 3C). The quantitative assessment of the changes in the level or phosphorylation of these proteins is shown in Fig. 3D).
Expression of a Constitutively Active Form of Akt (MyrAkt) Protects Cells from SCH66336-induced Apoptosis. The above experiments have implicated the Akt pathway in SCH66336-induced apoptosis indirectly. To further examine the role of Akt, we infected SqCC/Y1 cells with an adenoviral vector containing the constitutively active form of Akt1, namely MyrAkt, driven by the CMV promoter and tagged with HA. Fig. 4A shows that cells infected with the MyrAkt-HA-containing vector expressed the protein, as detected by the appearance of bands in Western blotting using either anti-HA antibodies (Fig. 4A1) or anti-Akt antibodies (Fig. 4A2). These bands could not be detected in cells infected with the control adenoviral vector (Ad5CMV). The constitutive activity of this protein was indicated by the increased phosphorylation of serine 9 of GSK-3β, an Akt substrate (Fig. 4A3) that had occurred without an increase in the level of the total GSK-3α/β protein (Fig. 4A4).

Having established that the activated Akt is expressed in the infected cells, we next determined whether this expression could protect the cells from the growth-suppressive effects of SCH66336. We found that cells infected with either $1 \times 10^5$ or $5 \times 10^5$ particles/cell were rescued from the effect of SCH66336 on cell survival compared with untreated cells or cells infected with control adenoviral vector (Fig. 4B). Furthermore, overexpression of MyrAkt protected the cells from SCH66336-induced apoptosis (Fig. 4C).

Discussion

Recent studies have indicated that some of the FTIs exhibit ras-independent antitumor activities (1). Because several FTIs are currently in clinical trials (1, 13), it is important to clarify their mechanism of action to use them optimally.

In the present study, we have demonstrated that the FTI SCH66336 can inhibit the growth of HNSCC cells in vitro at concentrations in the range between 0.1 and 8 μM, which are well below those reported to be achievable in vivo (about 8 μM) in mice given a single oral dose of 25 mg/kg SCH66336 (7). The effect of the higher doses was observed after 24 h, whereas lower doses required 3–5 days of incubation to exert their inhibitory effects. Cell cycle analysis revealed that SCH66336 increased the proportion of cells in the G2-M phase of the cycle. Anchorage-independent growth was also suppressed by SCH66336. After 3 days of treatment with 1 μM SCH66336, evidence for induction of apoptosis was clearly documented by several assays including cell cycle analysis (sub-G1 population increased), TUNEL, DNA laddering, activation of caspase-3, and PARP cleavage. These findings are the first report on the effects of SCH66336 on HNSCC cells. Some of our findings are similar to previous findings with other tumor cell types. For example, the inhibition of anchorage-dependent growth with cell accumulation in the G1 or G2-M phase has been observed in cell lines derived from breast, colon, pancreas, brain, and lung cancers (8, 14). However, our findings on induction of apoptosis in vitro are different from previous in vitro studies that SCH66336 was an effective inducer of apoptosis in our HNSCC cells when used as a single agent, whereas previous studies suggested that SCH66336 at doses similar to those that we have used cannot induce apoptosis unless it is combined with other death-promoting signals such as cell detachment (15), growth in low serum (16), or combination with cyclin-dependent kinase inhibitors (17).

The reason for the exquisite sensitivity of the SqCC/Y1 HNSCC cells to SCH66336 is not fully understood; however, it may be attributable to the induction of multiple proapoptotic effects by this agent. Specifically, SCH66336 decreased the levels of the antiapoptotic proteins Bcl-2 and Bcl-XL without affecting the level of the proapoptotic protein Bak resulting in a decrease in the ratio of the antiapoptotic:proapoptotic proteins. This effect alone could account for the proapoptotic effect of SCH66336. However, on top of this effect, SCH66336 also decreased the level of the proteins Akt1 and, to a lesser extent, Akt2, which are Ser/Thr PKB. These enzymes play...
important roles in cell proliferation and survival (18). PKB/Akt is activated in cells exposed to hormones, growth factors, and extracellular matrix components, which activate PI3K. PI3K activation produces phosphatidylinositol-3,4,5-trisphosphate PIP₃ (Ref. 3). This lipid acts as a second messenger to translocate PKB/Akt to the plasma membrane, in which it is phosphorylated by phosphoinositide-dependent kinase-1 (PDK-1) and possibly other kinases. PKB/Akt phosphorylates and regulates the function of many cellular proteins involved in processes that include apoptosis (caspase-9, Bad, TRAIL) and proliferation (p21, FKHR, GSK-3). This may explain why PKB/Akt is overexpressed or constitutively activated in many tumors (19, 20). Indeed, we found that all 10 of the HNSCC cell lines contained activated Akt (pAktS473), including high expressers (6 of 10 cell lines) and intermediate or low expressers (4 of 10 cell lines).

The consequences of the decrease in the Akt activity in SqCC/Y1 cells treated with SCH66336 for 12 h or more were lower levels of phosphorylated Akt substrates including pGSK-3β, pFRK, and pBad and each of these changes could also contribute to increased apoptosis. Because SCH66336 may exert multiple effects on a given cell, it may be difficult to elucidate its mechanism of action. Nonetheless, our study has demonstrated that a decrease in Akt activity appears to be the major proapoptotic mechanism in the SqCC/Y1 HNSCC cells because overexpression of a constitutively active Akt by infection with an adenoviral vector harboring MyrAkt controlled by a CMV promoter could rescue cells from the proapoptotic effect of SCH66336. The decrease in Akt level was also observed in HNSCC UMSCC38 (data not shown), suggesting that this effect of SCH66336 is not unique to SqCC/Y1 HNSCC cells.

Previous studies suggested that FTI inhibits PI³K/Akt-mediated growth factor- and adhesion-dependent survival pathways and induces apoptosis in human cancer cells that overexpress Akt via abrogation of the function of a putative farnesylated protein (1, 21). Endogenous Akt-2 levels, correlated with the ability of FTI-277 to induce apoptosis in H-Ras-transformed human cancer cells and overexpression of wild-type Akt2 but not of Akt1, sensitized cells to FTI-277 (22). Although these studies have tentatively linked Akt2 activity to the mechanism of FTI action, they are different from our findings insofar as we have shown that both Akt1 and Akt2 levels decreased and a constitutively active Akt1 was able to rescue the HNSCC cells from FTI SCH66336-induced apoptosis. This difference may be attributable to the difference in cell type used [NIH3T3 mouse fibroblasts in the Jiang et al. (22) study and HNSCC SqCC/Y1 in ours] or the different FTIs used [FTI-277 by Jiang et al. (22) and SCH66336 in our study]. One of the 10 HNSCC cell lines, 1438, had a high level of pAktS473 but was nonetheless resistant to SCH66336, which suggests that a high level of endogenous activated Akt may not be sufficient to confer sensitivity on the growth-inhibitory effects of SCH6636, at least in some cells.

The important functions that Akt plays in cell survival have made this enzyme an attractive molecular target for cancer drug development because inhibition of its activity induces apoptosis in a range of mammalian cancer cells. Therefore, our finding that SCH66336 can suppress of PKB/Akt activity suggests that this agent may be effective either alone or in combination with other anticancer drugs, for the treatment of HNSCC and possibly other tumors.

References

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